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Abstract: The blood-brain barrier (BBB) is a cellular barrier formed by specialized brain endothelial cells under the influence of astrocytes and pericytes. Among the several stress factors known to induce BBB breakdown, hypoxia is probably the most represented but also the least understood. Recent evidence of oxidative stress occurring during hypoxia/ischemia situation raises its possible contribution to barrier breakdown. In this study, we investigated the relevance of oxidative stress in hypoxia-induced barrier disruption. Prolonged hypoxic exposure induced radical oxygen species (ROS) formation and induced glutathione oxidation. Such effects were accentuated under extreme O(2) deprived environment. Pro-oxidant treatment significantly disrupted barrier function under normal conditions, whereas anti-oxidant treatment contributed to maintain better barrier function and cell survival in an O(2)-reduced environment. In addition, the endothelial response to oxidative stress appeared modulated by the presence of astrocytes and pericytes, thus explaining some of the beneficial contribution of these cells as previously described. Taken together, this study highlights the importance of oxidative stress signaling at the barrier. In addition, cells of the neurovascular compartment differentially modulate ROS levels and also regulate barrier function. Thus, use of radical scavengers may be useful to support barrier function following stroke injury.

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INVOLVEMENT OF OXIDATIVE STRESS IN HYPOXIA-INDUCED BLOOD-BRAIN BARRIER BREAKDOWN

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KEYWORDS: hypoxia; blood-brain barrier; astrocytes; pericytes; ROS; glutathione

HIGHLIGHTS:

- We investigated the relevance of oxidative stress in hypoxia-induced barrier disruption using an in vitro model.
- Prolonged hypoxic exposure induced reactive oxygen species formation and induced glutathione oxidation.
- Pro-oxidant treatment significantly disrupted barrier function under normal conditions.
- Anti-oxidant treatment maintained better barrier function and cell survival in an O_2-reduced environment.
- The endothelial response to oxidative stress was modulated by the presence of astrocytes and pericytes.

ABSTRACT

The blood-brain barrier (BBB) is a cellular barrier formed by specialized brain endothelial cells under the influence of astrocytes and pericytes. Among the several stress factors known to induce BBB breakdown, hypoxia is probably the most represented but also the least understood. Recent evidence of oxidative stress occurring during hypoxia/ischemia situation raises its possible contribution to barrier breakdown. In this study, we investigated the relevance of oxidative stress in hypoxia-induced barrier disruption.

Prolonged hypoxic exposure induced reactive oxygen species (ROS) formation and induced glutathione oxidation. Such effects were accentuated under extreme O_2 deprived environment. Pro-oxidant treatment significantly disrupted barrier function under normal conditions, whereas anti-oxidant treatment contributed to maintain better barrier function and cell survival in an O_2-reduced environment. In addition, the endothelial response to oxidative stress appeared modulated by the presence of astrocytes and pericytes, thus explaining some of the beneficial contribution of these cells as previously described.

Taken together, this study highlights the importance of oxidative stress signaling at the barrier. In addition, cells of the neurovascular compartment differentially modulate ROS levels and also regulate barrier function. Thus, use of reactive oxygen scavengers may be useful to support barrier function following stroke injury.

INTRODUCTION

The blood-brain barrier (BBB) constitutes a component of the neurovascular unit formed by specialized brain endothelial cells (ECs) surrounded by astrocytes, pericytes and neurons (Ogunshola, 2011; Rosenberg, 2012) . Due to the presence of important tight junction complexes and efflux transporters, these brain ECs play an important role as gatekeepers regulating central nervous system homeostasis.

The mammalian brain is extremely sensitive to changes in cellular homeostasis as a result of environmental or physiological insults and neurological diseases constitute one of the most challenging and devastating pathologies. Indeed the absence of reliable therapies to suppress or reverse their onset show that we need to better understand the complex mechanisms involved in progression of such diseases. Recent reviews highlight the contribution of an important cerebrovascular component to many pathologies including neurodegenerative diseases (Yang and Rosenberg, 2011; Zlokovic, 2011) and data suggests that BBB dysfunction is a cause or consequence of disease progression with overall negative outcomes.

Among the different stressors affecting the BBB integrity, two factors link impaired BBB and neurological diseases: hypoxia and oxidative stress.

Hypoxic/ischemic challenges (i.e. reduced oxygen and/or glucose delivery) cause severe and detrimental alterations in brain function, and can trigger neuronal cell death within minutes. Cerebral ischemia (or stroke) induces dramatic cerebral injury and a massive barrier disruption. Although different studies brought some crucial information on how hypoxic stress induces barrier disruption (Bauer et al., 2010; Vogel et al., 2007; Yan et al., 2011; Zhu et al., 2011), the mechanisms remain largely unclear.

Oxidative stress is defined as an imbalance between cellular pro-oxidants and anti-oxidants, resulting in excessive generation of reactive free radicals in the central nervous system (Mariani et al., 2005). Notably, presence of oxidative stress has been associated with many neurological diseases including stroke, neurodegenerative diseases and traumatic brain injury (Freeman and Keller, 2012). Although oxidative stress is known to induce BBB breakdown or paracellular permeability (Clanton, 2005; Lee et al., 2004; Lochhead et al., 2010; Yamagata et al., 2004) its contribution to hypoxia-induced barrier breakdown remains unknown.

In this study, we investigated the contribution of hypoxia-induced oxidative stress to barrier dysfunction in rat brain ECs.

MATERIALS AND METHODS

Cell culture & reagents

Rat brain endothelial cell line (RBE4) (Roux et al., 1994), primary rat brain astrocytes and pericytes were isolated and cultivated as described previously (Al Ahmad et al., 2009). Buthionine sulfoximine (BSO), desferrioxamine mesylate (DFO), reduced L-glutathione (GSH), *N*-acetylcysteine (NAC) and rotenone were purchased from Sigma-Aldrich (Buchs, Switzerland). Diphenyliodiunium (DPI) was purchased from Cayman Chemicals (Tallinn, Estonia). 5,6-carboxy-2',7'–dichlorofluorescein (DCF) was purchased from Invitrogen (Zug, Switzerland).

O_2 deprivation-reoxygenation experiments
Cells were exposed to either hypoxic (1% O_2) or near-anoxic (0.1%) conditions using a hypoxic chamber as previously described (Al Ahmad et al., 2009). Reoxygenation phase was achieved by replacing cell media and further expose cells to 21% O_2 for 24h.

Barrier function and cell viability
Barrier function was assessed by measuring transendothelial electrical resistance (TEER) as previously described (Al Ahmad et al., 2009). Cell viability was assayed using an ADAM fluorescent cell counter (Witec AG, Littau, Switzerland). Living cells were expressed as a percentage of total cell count.

Cellular ROS generation and GSH measurements
ROS formation was quantified by fluorometry techniques based on DCF oxidation, whereas GSH measurement was obtained using colorimetry technique with protocols previously published in the literature (Bogdanova et al., 2005; Petrushanko et al., 2006).

Statistical analysis
All results are expressed as mean ± SD from three independent experiments. Statistical significance was assessed by analysis of variance using appropriate tests. A *p*-value below 0.05 was considered significant.

RESULTS
We firstly monitored oxidative stress in RBE4 cells during O_2 deprivation insult using DCF, a fluorescent dye sensitive to ROS compounds (Figure 1A). Hypoxic stress was not efficient to generate detectable ROS formation in RBE4 monolayers, as we noted no differences in DCF fluorescence compared to normoxia. However near-anoxic conditions strongly induced ROS formation, as we noted a significant increase in DCF fluorescence. Following such observation, we measured reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio in cells exposed to hypoxia or near-anoxia for 24h (Fig.1B). Under hypoxic stress, such ratio was significantly increased compared to control condition, suggesting an up-regulation in GSH synthesis. However, under near-anoxic conditions the ratio was significantly lower suggesting the onset of oxidative stress in RBE4 monolayers and correlating with the DCF data.
As O_2 deprivation induced ROS formation in RBE4 monolayers, we investigated the impact of oxidative stress on barrier function in normoxic cells. Presence of 200 µM BSO (an inhibitor of GSH synthesis) transiently decreased TEER (Fig.2A). However, the presence of 10µM rotenone significantly decreased TEER compared to control condition. Such decrease in barrier function was unlikely related to cell-death mediated mechanisms, as no cell death was noted after 24h (Fig.2B).
To better correlate hypoxia-induced barrier breakdown and increase oxidative stress, we investigated the effect of anti-oxidant treatment on barrier function using 5mM NAC, 100µM DFO or 10µM DPI as anti-oxidant reagents (Fig.3). Under hypoxic condition (Fig.3A), presence of NAC, DFO or DPI did not significantly affect TEER values compared to control during acute exposure (less than 8h). DPI treatment partially

maintained barrier function already by 24h, whereas DFO- or NAC-treated cells showed improvement only after 48h. Under near-anoxic condition (Fig.3B), all but DFO treatment allowed RBE4 cells to maintain higher TEER than vehicle-treated group from 6h to 48h. Again, no significant improvements were noted following reoxygenation.
Notably cell survival following prolonged (48h) hypoxic injury was improved by all three treatments, whereas DPI treated-group only showed significant improvement in near-anoxic cells (Fig.4A). Finally, we investigated the effect of astrocyte and pericyte co-cultures on the GSH ratio in RBE4 cells (Fig.4B). After 24h of normoxia, no significant differences were observed between monocultures and co-cultures. However, after 24h of hypoxia, pericyte co-cultures showed a three-fold decrease in GSH/GSSG ratio whereas astrocyte co-cultures showed no differences compared to monocultues.

DISCUSSION

O_2 deprivation injury constitutes one of the most challenging pathophysiological mechanisms leading to BBB breakdown. Although some of the cellular and molecular mechanisms triggering such breakdown are better understood, existence of an therapeutical arsenal capable of restoring BBB function remains largely missing. Recent description of oxidative stress occurring under O_2 deprivation insult (Chandel et al., 1998) raises the possible contribution of ROS signaling to BBB breakdown. In this study, we provide evidence of the involvement of ROS in RBE4 barrier function disruption during hypoxia, a condition known to induce vascular leakage in ECs (Al Ahmad et al., 2009). We demonstrate that oxidative stress significantly contributes to barrier breakdown due to the fact that artificial generation of ROS increased ECs barrier breakdown, whereas treatment of RBE4 monolayers with anti-oxidants during O_2 deprivation stress resulted in overall improvement of both barrier function and cell survival. Although we did not identify the specific chemical nature of such pro-oxidant species, we can speculate that the effects are likely driven through different species. H_2O_2 formation through Fenton's reaction is known to induce barrier leakage (Kim et al., 2008; Lee et al., 2004; Yamagata et al., 2004). However inhibition of H_2O_2 by DFO (an iron chelator) was beneficial only under hypoxia. On the other hand, DPI (an NADPH oxidase inhibitor) or NAC (glutathione analog) were efficient both under hypoxic and near-anoxic treatment suggesting that ROS products distinct from H_2O_2 may occur under such severe conditions.
Among the different protective mechanisms developed by mammalian cells to fight against such oxidative stress, glutathione is certainly the most prominent (Dickinson et al., 2003). Using GSH/GSSG ratio as a marker of oxidative stress, we observed that hypoxic cells showed the highest ratio whereas near-anoxic cells showed the lowest ratio suggesting that an impaired redox cellular homeostasis may contribute to hypoxia-driven barrier breakdown. Such impairment appeared crucial during oxidative stress as artificial supplementation of reduced glutathione (using NAC) improved both barrier function and cell survival. On the other hand, a direct inhibition of ROS generation by DPI showed the most potent effect in maintaining barrier function and improving cell survival, whereas attempts to selectively inhibit only one ROS by-product (H_2O_2 by DFO) appeared limited. Thus, a combined inhibition approach (by simultaneously inhibiting ROS generation and increasing reduced glutathione bioavailability) may help in reducing BBB leakage and improving its restoration.

As the BBB is a multicellular unit, it is important to consider the potential modulatory effects of astrocytes and pericytes on EC response to oxidative stress. Previous studies highlighted the ability of astrocytes to "buffer" oxidative stress when co-cultured with brain ECs (Schroeter et al., 1999), whereas a recent study highlighted the high susceptibility of brain pericytes to oxidative stress (Price et al., 2012). Furthermore, we previously demonstrated that astrocytes but not pericytes maintained better barrier function following mild hypoxia (Al Ahmad et al., 2009). Using glutathione ratio to assess oxidative stress, we noted a significant decrease in the pericyte co-culture ratio after 24 hours of hypoxia compared to monocultures and astrocyte co-cultures. This data agrees with a previous study that highlighted the negative impact of EC-generated ROS on pericyte function (Amano et al., 2005). Our observations may partially explain the differential response observed between astrocyte and pericyte co-cultures on barrier function and raises the question whether pericytes respond to oxidative stress only under severe conditions. Based on our current data, we can speculate that the generation of distinct ROS by-products by EC monolayers may condition the cellular response of astrocytes and pericytes to oxidative stress. Thus further investigations are necessary to shed light on the cellular mechanisms underlying astrocyte and pericyte response to such oxidative stress.

Finally, the concomitant presence of oxidative stress and O_2 deprivation raises the question of whether hypoxia-induced factor (HIF) pathways modulate the cellular response to hypoxia-induced oxidative stress (Fandrey and Gassmann, 2009). It has been suggested that increased oxidative stress may increase HIF-1 activity (Tajima et al., 2009), which in turn may decrease GSH biosynthesis (Jackson and Gupta, 2010). Such hypothesis requires further investigation in order to understand the contribution of HIF-1 to changes in GSH content in RBE4 monolayers during O_2 deprivation injury.

In summary this study provides insight into the effect of oxidative stress on BBB function during hypoxia insult and shed a new light on the roles played by astrocytes and pericytes regarding such stress. This study suggests that the generation of reactive oxygen species is somewhat proportional to O_2 deprivation severity and contributes to hypoxia-induced barrier breakdown. The ability of brain ECs to cope with stress endogenously (through the glutathione metabolism) or by involving neighboring cells (astrocytes and pericytes) determines whether the barrier maintains its function or is compromised.

As recently suggested (Freeman and Keller, 2012; Ogunshola, 2011) the BBB likely constitutes the first interface of the central nervous system confronted by oxidative stress following stroke or indeed any cerebrovascular disease and an impaired response, or disruption of function, would directly impact neuronal integrity and survival. Thus, a better understanding of how the BBB copes with oxidative stress may represent a promising target to simultaneously fight ongoing oxidative stress at the injured site but also foster a restoration of the barrier function following acute and chronic cerebrovascular diseases.

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FIGURES LEGEND

Figure 1: Near-anoxia induces excessive ROS formation in RBE4 monolayers.

A: ROS production was measured by DCF fluorescence in cells pre-incubated under hypoxic or near-anoxic condition. 1 hour before the end of experiments, 10µM DCFDA was added. Cells were lyzed to measure cellular DCF fluorescence. n=3, ** $P<0.01$ vs. 21% O_2, ## $P<0.01$ vs. 1% O_2. B: Reduced GSH and oxidized GSH (GSSG) were measured in cells exposed for 24h to hypoxia or near-anoxia. n=3, *$P<0.05$ and ** $P<0.01$ vs. 21% O_2, ## $P<0.01$ vs. 1% O_2.

Figure 2: Pro-oxidative stress induces barrier leakage under normoxic conditions.

A: TEER measurements were performed in RBE4 monolayers exposed to 200µM BSO or 10µM rotenone. Vehicle group (0.1% DMSO) was used as a timeline control. n=3, *$P<0.05$ and ** $P<0.01$ vs. vehicle. B: Cell survival was assessed in cells exposed for 48h to BSO or rotenone. Number of living cells was normalized to total cell count. n=3.

Figure 3: Anti-oxidant treatment partially prevent hypoxia-induced BBB breakdown

TEER measurements were performed in RBE4 monolayers exposed to hypoxia (A) or near-anoxia (B). 100µM DFO, 10µM DPI or 5mM NAC were added 1h before experimental start. Vehicle group (0.1% DMSO) was used as a timeline control. Reoxygenation was performed by incubating cells in fresh medium at 21% O_2 for 24h. n=3, *$P<0.05$ and **$P<0.01$ vs. vehicle.

Figure 4: Effect of anti-oxidant treatment and co-culturing on ECs cell survival and redox status

A: Cell survival was assessed in cells exposed for 48h to hypoxia or near-anoxia in presence of DFO, DPI or NAC. Living cells were normalized over total cell count. n=3.

*P<0.05 versus vehicle-treated group. B: Effect of co-culturing astrocytes and pericytes on ECs redox status. Co-cultures were incubated for 24h either under normoxic (21% O_2) or hypoxic (1% O_2) condition. n=3, *P<0.05 and **P<0.01 vs. 21% O_2.

Figure 1

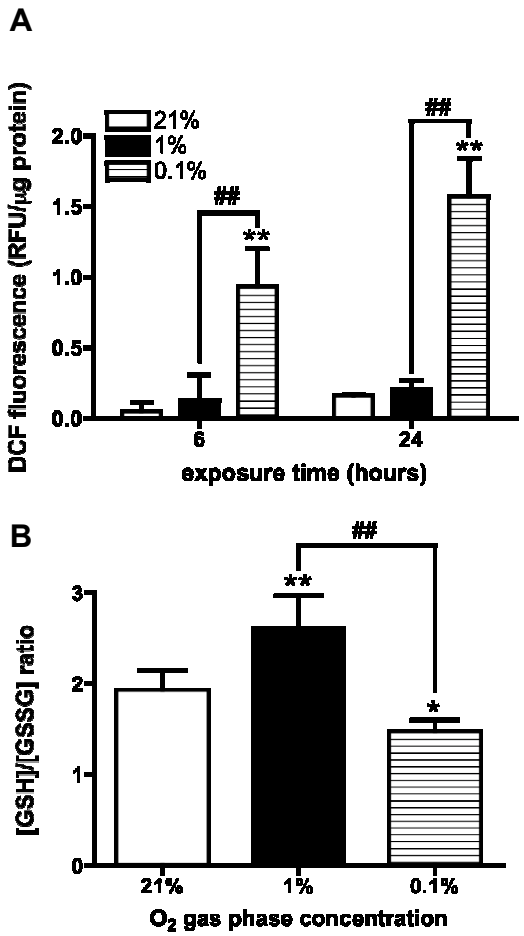

Figure 2

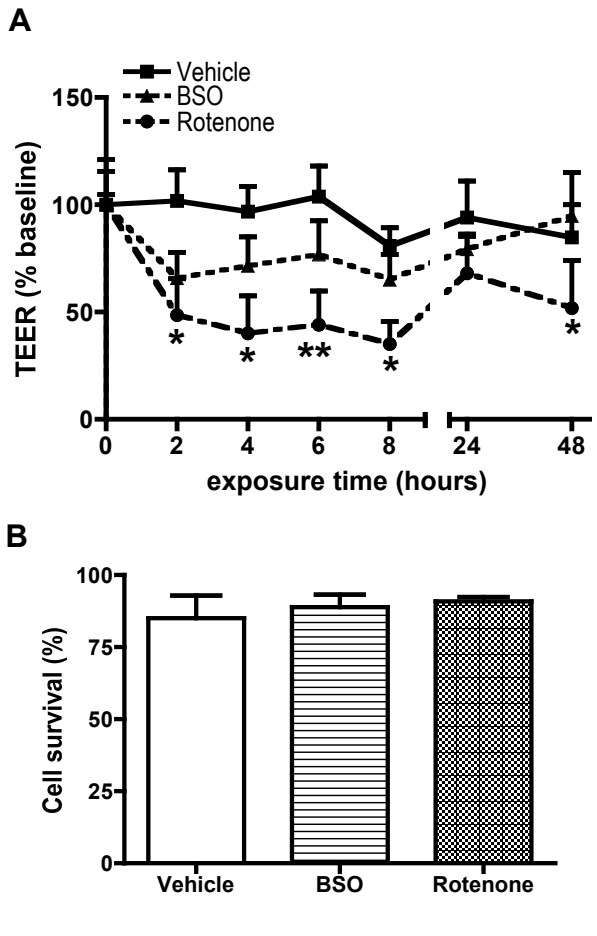

Figure 3

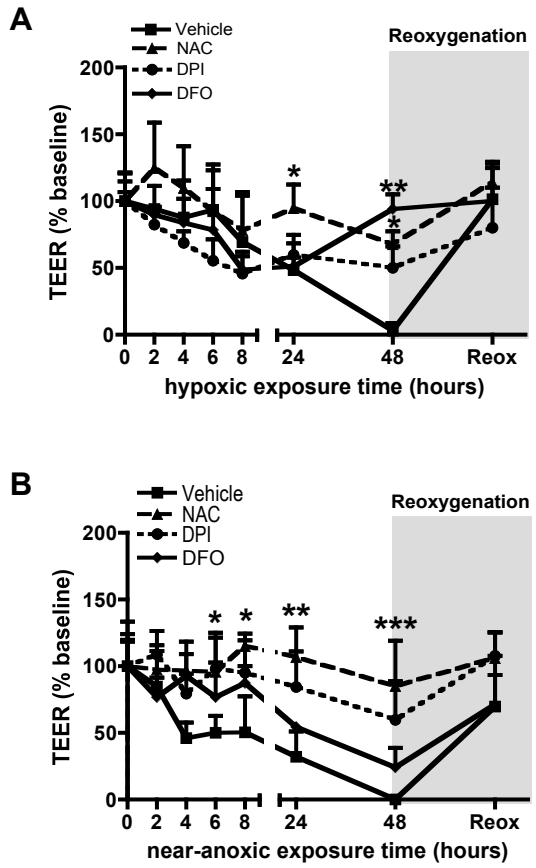

Figure 4